Host Responses to Leptospiral Infections

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LIST OF USEFUL ABBREVEATIONS

AP | alkaline phosphatase
APTT | activated partial thromboplastin time
ALT | alanine transaminase
AST | aspartate transaminase
CD | cluster of differentiation
CMP | common myeloid progenitor
ECP | eosinophil cationic protein
EDN | eosinophil derived neurotoxin
ELISA | enzyme-linked immunosorbent assay
EPO | eosinophil peroxidase
EMJH | Ellinghausen-McCullough-Johnson-Harris
GGT | Gamma-glutamyl transferase
G-CSF | granulocyte colony stimulating factor
GM-CSF | granulocyte macrophage colony stimulating factor
INFγ | gamma interferon
HSC | haemopoietic stem cells
LDH | lactate dehydrogenase
IL-1 | interleukin 1
IL-2 | interleukin 2
IL-3 | interleukin 3
IL-4 | interleukin 4
IL-5 | interleukin 5
IL-6 | interleukin 6
IL-7 | interleukin 7
IL-8 | interleukin 8
IL-9 | interleukin 9
IL-10 | interleukin 10
IL-11 | interleukin 11
MAC | membrane attack complex
MAT | microscopic agglutination test
MBL | mannann-binding lectin
MBP | major basic protein
M-CSF | macrophage colony stimulating factor
MEP | megakaryocyte-erythroid progenitor
MHC-1 | major histocompatibility complex class 1
MHC-2 | major histocompatibility complex class 2
NET | neutrophil extracellular traps
NK | natural killer cell
PT | prothrombin time
RBC | red blood cell
RCC | red cell count
SCF | stem cell factor
WBC | white blood cell
WCC  white cell count
LIST OF KEY WORDS

*Leptospira*, leptospirosis, host responses, laboratory markers, haematology, clinical chemistry, immunology, tropical medicine, lymphopenia, hypomagnesemia, Goodpasture’s syndrome, type 4 collagen.
Declaration

I declare that this thesis is my own work, accept where attribution has been indicated and has not been submitted in any form for another degree or diploma at any University or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Scott Craig
11/09/2010
ABSTRACT

Leptospires, the etiological agent of leptospirosis, are aggressive ubiquitous bacteria with a worldwide distribution, causing a spectrum of disease ranging from a mild influenza like illness to Weil’s disease which manifests itself in multi organ failure. Studies delineating host responses to these bacteria are limited as is the current understanding of the pathology engendered by these pathogens. The overarching aim of this thesis is to explore and delineate targeted host responses in patients with leptospirosis.

The first specific aim of the thesis was to resolve the paradox in the research literature in regards to whether lymphopenia is a common phenomenon in leptospirosis patients and whether or not lymphopenia is Leptospira serovar dependent. To investigate lymphopenia (< 1.2 x 10⁹ lymphocytes / L) in leptospirosis, haematological results from 258 patients diagnosed with leptospirosis at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis in Queensland, Australia were analysed to determine the frequency of lymphopenia and determine if lymphopenia is serovar dependant. The results revealed that lymphopenia during leptospirosis appears common across the majority of pathogenic serovars routinely screened for at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis. The possible exception is L. interrogans serovar Copenhageni.
The second aim of the thesis was an extension of the first aim. The second aim of this thesis was to determine if lymphopenia is commonly observed in not just the acute phase but also the immune phase of the disease. To investigate this aim lymphocyte counts from 57 leptospirosis patients in different phases of the disease were interrogated for the incidence of lymphopenia. This study revealed a higher incidence of lymphopenia in the acute phase than the immune phase of the disease and suggested that the phase of the leptospiral infection may affect patient lymphocyte counts.

The third aim of this thesis was to examine any differences in laboratory markers and to differentiate those with an uncomplicated episode of leptospirosis from those who were admitted to an intensive care unit with severe disease. This was to identify leptospirosis patients who need more aggressive treatment regimen/s directed toward them. To facilitate this aim the haematological and clinical chemistry results from patients who survived severe leptospirosis were compared to those who recovered from an unremarkable episode of leptospirosis. The results from this investigation suggest that when leptospirosis is suspected, patients presenting with any of the following; serum urea greater than 7.1 mmol/L, creatinine greater than 120 mmol/L, albumin less than 35 g/L, haemoglobin less than 135 g/L, haematocrit below 0.36, red cell count less than 4.5 x 10⁹/L, platelet count less than 140 x 10⁹/L, a white cell count greater than 11 x 10⁹/L and neutrophil count greater than 8 x 10⁹/L may need to be closely monitored and have aggressive treatment regimes directed toward them.
The fourth aim of this thesis was to monitor the magnesium status in leptospirosis patients during the acute phase (first 10 days of infection) to determine if they develop hypomagnesaemia given the paradox in the research literature were some studies had observed it and others had not. To facilitate this aim the serum magnesium status of 15 patients with leptospirosis were monitored for 10 days. The results showed that more attention needs to be paid to the serum magnesium status in leptospirosis patients as 14 of the 15 patients examined displayed hypomagnesaemia during the acute phase of the disease.

The fifth aim of this thesis was to determine if laboratory markers in a second consecutive infection with a different serovar of leptospires are markedly different. The case study presented in this thesis, of a patient who had two confirmed (by culture isolation) leptospiral infections, revealed that in a secondary infection lymphopenia and thrombocytopenia were evidenced and liver function tests (ALT and AST) were within the normal reference range. This study also revealed that serological identification may be difficult because of paradoxical reactions or original antigenic sin.

Finally, in the investigation of host responses to leptospiral infections, this thesis examined if leptospirosis patients produce auto antibodies to type 4 collagen (the Goodpasture’s Syndrome antigen). To facilitate this aim reactive leptospirosis patient serum from 40 patients was assayed in a commercial ELISA. The results of the investigation of leptospires as etiological agents of Goodpasture’s syndrome
revealed that the host does not produce auto antibodies directed toward type 4 collagen.
Chapter 1

Introduction
The first modern observation of leptospirosis was recorded in a number of Napoleon’s troops during their defeat of the Turkish armies at Heliopolis in 1800. During the siege of Cairo, troops suffered ‘fievre jaune’ now known as Weil’s disease, the severe form of leptospirosis (Faine et al., 1999). It would take another ninety years before icteric leptospirosis with renal failure was first credibly reported by Adolf Weil in his seminal work “Ueber eine eigentumliche, mit Milztumor, Icterus und Nephritis einhergehende akute Infektionskrankheit. (Concerning a characteristic infectious diseases, accompanied by splenomegaly, jaundice and nephritis) (Weil, 1886).

Leptospirosis is an aggressive zoonoses caused by bacteria of the genus *Leptospira* (Figure 1.1). Leptospires are ubiquitous pathogens, with a world-wide distribution, causing a spectrum of disease ranging from a mild influenza-like illness to Weil’s disease—which manifests itself in multi-organ failure (WHO, 2003). Leptospires are 6 to 20 μm in length and 0.1 to 0.2 μm in diameter, have optimal growth at 30 °C, are obligate aerobes and utilize long-chain fatty acids as their carbon source (Levett, 2001).

Leptospires enter the host via cuts and abrasions in the skin or acquire direct access into the blood or lymphatics of the host via the conjunctiva or lungs following the inhalation of aerosols. Inhalation of water or aerosols also may result in infection via the mucosal membranes of the respiratory tract (Faine, et al., 1999; Levett, 2001). Only rarely has the direct transmission between humans been
demonstrated (Harrison & Fitzgerald, 1988). Once in the blood, leptospires are capable of circulating to all tissues.

Leptospires evading phagocytic cells of the reticuloendothelial system grow in an exponential manner doubling every eight hours (Faine et al., 1999). There is evidence that phagocytosed leptospires do not survive long in the interior milieu of the phagocyte (Vinh, Adler & Faine, 1982; Wang et al., 1984). Virulent strains have the ability to attenuate the phagocytic responses by activating apoptosis in the macrophage (Merien, Baranton & Perolat, 1997).

Figure 1.1 Scanning electron micrograph of Leptospira. Copyright B Harrower, Queensland Health, 2007. Reproduced with permission.
The minimum infectious dose leading to leptospirosis is thought to be small however the length of time of the incubation period is assumed to be inversely correlated with the size of the infecting dose. For example, a high infecting dose may engender a short incubation period when compared to a low infecting dose. Interestingly, small doses (1-3 organisms) may result in prolonged incubation times which may extend into the immune phase resulting in a mild or even sub clinical infection (Faine et al., 1999).

Following the initial incubation period, the host enters the acute phase of the disease that usually lasts seven to ten days. Clinically, during the acute phase, patients typically present with headache, fever, excruciating myalgia and arthralgia and sometimes rigours, vomiting, photophobia and a mucosal rash. These symptoms closely mimic those of other tropical infectious diseases making a diagnosis of leptospirosis difficult. Haemoptysis, hypotension and bradycardia are also common presentations. Hepatosplenomegaly, jaundice (produced as a result of hepatocellular damage, increased erythrocyte destruction and the resulting increase in circulating haemoglobin and bilirubin), renal failure, liver failure and acute respiratory distress are common features of the more acute form of the disease known as Weil’s disease or icteric leptospirosis (Sutliff, Shepard & Dunham, 1953; Solbrig, Sher & Kula, 1987; Faine et al., 1999, Levett, 2001). Following the acute phase, patients enter the immune phase where immunoglobulins, specific for leptospires, are produced to resolve the infection (Levett, 2001).
Acute phase diagnosis can be performed using methods such as IgM specific enzyme-linked immunosorbent assays (ELISA) (Milner et al., 1985) lateral flow enzyme immunosorbent assay (Smits et al., 2001) and the culture of the blood, cerebrospinal fluid, or urine in Ellinghausen-McCullough-Johnson-Harris (EMJH) media (Faine et al., 1999). PCR and real time PCR technologies have also emerged with accuracy in terms of sensitivity and specificity (Bharti et al., 2003; Slack et al., 2007).

Diagnosis during the immune phase relies on the Microscopic Agglutination Test (MAT) (Faine, 1982). The MAT detects the presence of antibodies specific to leptospires using a panel of antigens in the form of inactivated or live organisms representing the serogroups or serovars indigenous to the given geographical location. In Australia, serological confirmation requires a 4-fold or greater rise in agglutination titre between the acute and convalescent (immune phase) sera, obtained at least 10 to 14 days apart. A single Leptospira MAT ≥ 400 supported by a positive ELISA IgM result is also indicative of a recent infection. The end point of the MAT reaction is deemed as the dilution of serum, which facilitates 50% agglutination, leaving 50% of leptospires free when observed under dark-field microscopy (ILS, 1984).

Treatment regimens vary and involve the correction of electrolyte abnormalities and hypovolemia (Faine, 1994). Respiratory, renal and haemodynamic support may be indicated. Patients with uncomplicated leptospirosis may be treated with doxycycline (100 mg orally, twice daily for 7 days) (McClain et al., 1984).
Patients hospitalised for leptospirosis presenting with hepatic failure, renal failure or severe neurological disturbances, may be treated with intravenous penicillin G (1.5 million U every 6 hours for 7 days) (Watt et al., 1988).

Although members of the genus *Leptospira* are a major cause of mortality and morbidity in tropical and subtropical regions of the world, progress has been slow in delineating the mechanisms underpinning pathogenesis. Clearly, valuable information and clues to mechanisms of pathogenesis can be gained by analyses of blood, examining both biochemical and haematological markers. Such analyses can be thought of as investigations into the host responses to leptospiral infections, and form a central core of investigations reported in this thesis. Because of this, the following sections are designed to give an overview of human haematology, immunology and biochemistry in the context of responses to challenges by infectious agents as these provide pivotal entities capable of responding to an infection.

### 1. Haematology and Immunology

#### 1.1 Haemopoiesis

Haemopoiesis or the production of blood cells occurs primarily in the microenvironment of the bone marrow. The microenvironment of the bone marrow primarily consists of haemopoietic cells, macrophages, fibroblasts, fat cells, collagen and a matrix consisting of collagen, and ground substance (Gordon-Smith, 2009a). In adults, haemopoiesis occurs in the axial skeleton, sternum, scapula, pelvis, ribs, sternum, pelvis and skeletal vertebrae. The proliferation, differentiation and
apoptosis of bone marrow cells are orchestrated biochemically by a number of cytokines (Figure 1.2 and Table 1.1) (Gordon-Smith, 2009a).

Figure 1.2 A general model of hematopoiesis (Adapted from Kaushansky, 2006).

Legend: HSC (hematopoietic stem cell), CLP (common lymphoid progenitor), CMP (common myeloid progenitor), TNKs (T cells and natural killer cells), GMs (granulocytes and macrophages GMs), MEPs (megakaryocytes and erythroid cells). BCP’s (B cells), NKP’s (NK cells), TCPs (T cells), GPs (granulocytes), MPs (monocytes), EPs (erythrocytes) and MkPs (megakaryocytes).
Table 1.1 Haemopoietic Growth Factors (Adapted from McKenzie, 1996).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Target Cell</th>
<th>Production Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>Monocytes</td>
<td>Endothelial Cells, Monocytes, Fibroblasts</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocytes, Megakaryocytes, Stem Cells, Erythrocytes</td>
<td>T Cells, Endothelial Cells, Fibroblasts</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocytes, Macrophages, Endothelial Cells, Fibroblasts</td>
<td>Endothelial Cells, Monocytes</td>
</tr>
<tr>
<td>IL-1</td>
<td>Stimulates expression of growth factor by other cells.</td>
<td>Monocytes, Macrophages, Endothelial Cells</td>
</tr>
<tr>
<td>IL-2</td>
<td>T and B lymphocytes</td>
<td>T Cells</td>
</tr>
<tr>
<td>IL-3</td>
<td>Granulocytes, Erythroid Cells, MPPC,</td>
<td>T Cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>B and T Cells</td>
<td>T Cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>B Cells, Erythroid Precursors</td>
<td>T Cells</td>
</tr>
<tr>
<td>IL-6</td>
<td>B and T Cells, Granulocyte and Erythroid Precursors, Macrophages</td>
<td>Fibroblasts, Leukocytes, Epithelial Cells</td>
</tr>
<tr>
<td>IL-7</td>
<td>B Cells</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>IL-8</td>
<td>T Cells and Neutrophils</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>IL-9</td>
<td>Granulocyte and Erythroid Precursors</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>IL-11</td>
<td>B and T Cells, Granulocyte and Erythroid Precursors, Macrophages and Megakaryocytes</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Erythropoietin Stem Cell Factor (SCF)</td>
<td>Erythroid Precursors</td>
<td>Kidney, Liver</td>
</tr>
<tr>
<td></td>
<td>Primitive Progenitors</td>
<td>Not Identified</td>
</tr>
</tbody>
</table>

Cytokines are responsible for the activation of multi-potent progenitor haemopoietic stem cells (HSC) and facilitate the development of multi-potent progenitor cells which can differentiate into cells of either the myeloid or lymphoid series. The HSC can give rise to a common myeloid progenitor (CMP) that can differentiate into either a megakaryocyte-erythroid progenitor (MEP) and then go on...
to develop into erythrocytes (red blood cells) or megakaryocytes. The CMP can also a granulocyte-monoocyte progenitor resulting in the formation of granulocytes (neutrophils, eosinophils and basophils) or monocytes. The HSC can alternatively give rise to a common lymphoid progenitor that can go on to become a dendritic cell or a T, B or Natural Killer (NK) lymphocyte. Each of the cells can be differentiated immunophenotypically by cluster of differentiation (CD) markers on the cell surface (Kaushansky, 2006). An absence of stimulatory cytokine signals arrests proliferation and differentiation and may facilitate apoptosis resulting in the biochemical execution of the cell (Gordon-Smith, 2009a).

1.2 Neutrophils

Neutrophils (Figure 1.3) are the most abundant leukocyte circulating in the peripheral blood. The normal number varies between 2 and 8 x 10^9 / L. Neutrophilia is the term used to describe the condition when the number of neutrophils seen in a peripheral blood smear is > 8 x 10^9 / L. Neutropenia is observed when the number of neutrophils seen in a peripheral blood smear is < 2 x 10^9 / L (McKenzie, 1996).
Neutrophils are pivotal in the hosts defence. Neutrophils migrate to the site of inflammation or infection, phagocytose microorganisms and damaged host cells and produce a number toxic products and enzymes that have a microbicidal effect (Figure 1.4).
**Figure 1.4.** Neutrophil microbicidal products that attack microbes. Microbicidal products arise from most compartments of the neutrophil: azurophilic granules (also known as primary granules), specific granules (also known as secondary granules), and tertiary granules, plasma and phagosomal membranes, the nucleus and the cytosol. BPI, bactericidal permeability increasing protein; H2O2, hydrogen peroxide; HOBr, hypobromous acid; HOCl, hypochlorous acid; HOI, hypoiodosus acid; MMP, matrix metalloproteinase; \( {\cdot}O_2 \), singlet oxygen; \( O_2^- \), superoxide; \( O_3 \), ozone; \( \cdot\)OH, hydroxyl radical; Phox, phagocyte oxidase (Adapted from Nathan, 2006).

Recently, it has been postulated that neutrophils inform and shape immune responses via their secreted products and cell-cell contact (Nathan, 2006) (Figure 1.5).
Figure 1.5. Neutrophils interact with other leukocytes (Adapted from Nathan 2006).
Research also indicates that, in septic patients, platelets aggregate and bind via Toll-like receptor 4 molecules directly to neutrophils (Ma & Kubes, 2008). This binding may facilitate the release of chromatin and azurophilic and gelatinase granules to form neutrophil extracellular traps (NET’s) in the microvasculature, where organisms can be trapped and destroyed (Figure 1.6).

Figure 1.6. NET formation allows a greater number bacteria to be trapped within the microvasculature where they can be killed and destroyed (Adapted from Ma & Kubes, 2008).
1.3 Lymphocytes

Lymphocytes are (Figure 1.7) the second-most abundant leukocyte circulating in the peripheral blood. The normal number varies between 1.2 and $4 \times 10^9 / L$. Lymphocytosis is the term used to describe the condition when the number of lymphocytes seen in a peripheral blood smear is $> 4 \times 10^9 / L$. Lymphopenia is observed when the number of lymphocytes seen in a peripheral blood smear is $< 1.5 \times 10^9 / L$. (Carrick & Begg, 2008; McKenzie, 1996). Lymphocytes as a group of leukocytes are divided into two groups: T and B lymphocytes. Both classes of lymphocytes are derived from the bone marrow stem cells however, T cells undergo further processing and maturation in the thymus due to the thymosin produced by the epithelial cells in the thymus. T lymphocytes are differentiated in the thymus into either T-helper (Th/CD4+) or T-cytotoxic (Tc/CD8+) cells. Th/CD4+ cells can be further divided into Th1 and Th2 cells according to the cytokines that each produce. Th1 cells produce cytokines such as gamma interferon (INFγ), and interleukin 2 (IL-2) involved in cell mediated immunity. Th2 cells produce cytokines such as interleukins; 3 (IL-3), 4 (IL-4), 5 (IL5), 6 (IL-6) and 10 (IL-10) that underpin the production of immunoglobulin / antibodies (Carrick & Begg, 2008; McKenzie, 1996). There is evidence that the Th1 / Th2 paradigm is limited and that additional paradigms are evolving (Chaouat et al., 2004). Th/CD4+ cells recognise antigenic peptides presented on major histocompatibility complex class 1 molecules (MHC-1). Tc/CD8+ cells recognise antigenic peptides presented on major histocompatibility
complex class 2 molecules (MHC-2). The activation of cytotoxic T cells by IL-2 produced by Th/CD4+ cells or the cytotoxic T cell precursors themselves results in the release of cytotoxic proteins such as perforin and granzymes and the expression of FAS ligand on the Tc/CD8+ cell resulting in the biochemical execution or apoptosis of the target cell (Figure 1.8).

**Figure 1.7.** Giemsa stain of peripheral blood lymphocyte (Adapted from McKenzie, 1996).
Natural killer (NK) cells are large granular lymphocytes that make up approximately 10% of the lymphocyte population. The granules and cytotoxic activity of these cells are similar to the Tc/CD8+ cells, however unlike Tc/CD8+ cells, which require activation before becoming granular, NK cells are constitutively

**Figure 1.8.** Three pathways leading to apoptosis. Caspases that orchestrate cell death can be activated by granzyme B, and the effector molecules activated by the binding of FAS and TRAIL to their receptors. Cytochrome C release from the mitochondria may also initiate caspase activation (Adapted from Huessler et al., 2001).
NK cells are not MHC restricted and instead bind to the carbohydrate moieties of glycoproteins in infected or tumor cells via their NKR-P1 receptor (Versteeg, 1992; Berke, 1995; Kuby, 1997).

B lymphocytes have membrane bound antibodies as antigen binding receptors and are the cells primarily involved in humoral / antibody immune responses. After antigen binds to the membrane bound antibodies it is internalised, processed and presented on MHC-2 molecules. Th/CD4+ cells then bind and secrete cytokines such as IL-2, IL-4, IL-5 and IL-6 to facilitate the development of antibody secreting plasma cells and memory B cells that will respond quickly to future infections (Figure 1.9) (Kuby, 1997). Antibodies produced by B cells when bound to antigen help activate the complement classical pathway cascade. The complement cascade results in the formation of proteins such as C5a which acts as chemoattractant for neutrophils and C3b which opsonizes microbes to enhance phagocytosis and the presentation of antigen to lymphocytes. The generation of complement factor C5b results in the activation of the membrane attack complex (MAC) which results in damage and ultimately lysis of pathogens (Tomlinson, 1993). In addition to classical antibody-antigen complexes, the complement cascade can be activated by the mannann-binding lectin (MBL) pathway. In this pathway MBL binds to microbial carbohydrates such as mannose and then activates complement factors resulting in the formation of the MAC, C3b and C5a. A third pathway known as the alternative pathway uses C3, factor B, factor D and properdin to amplify the effects of the classical pathway (Figure 1.10) (Janway et al., 1999; Carmichael & Wills, 2005).
Figure 1.9. T cell interactions in immune responses (Adapted from Kuby, 1997).
Figure 1.10. Complement overview (Adapted from Janway et al., 1999).

1.4 Monocytes

Monocytes (Figure 1.11) make up approximately 4% to 10% of the total leukocytes in circulation. Monocytes leave the circulation and reside in numerous tissues as macrophages. Monocytes / macrophages ingest and kill microbes and present antigens via their MHC-2 molecules and produce cytokines to facilitate lymphocyte responses. Monocytes/ macrophages are potent scavengers, phagocytosing cellular debris, they ingest activated clotting factors limiting
Figure 1.11. Giemsa stain of peripheral blood monocyte (Adapted from Hoffbrand & Pettit, 1999).

Coagulation and they ingest antigen-antibody complexes (Mckenzie, 1996; Gorden-Smith 2009b).

1.5 Eosinophils

Eosinophils (Figure 1.12) account for 1% to 3% of circulating leukocytes. These leukocytes are granulated with granules containing four major proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN). Other granules include contain enzymes such as acid phosphatase, glycuronidase, cathepsins, aryl-sulphatase, histaminase, collagenase and catalase (McKenzie, 1996). Eosinophil numbers increase in allergic reactions and in response to parasitic infections. Sustained eosinophilia (increased
number of eosinophils) may engender a hypereosinophilic syndrome, the features of which include fever, weight loss, splenomegaly and thromboembolic episodes (Gorden-Smith, 2009b).

![Figure 1.12. Giemsa stain of peripheral blood eosinophil (Adapted from McKenzie, 1996).](image)

**1.6 Basophils**

Basophils (Figure 1.13) account for 0% to 1% of circulating leukocytes. These leukocytes are also associated with allergic responses and parasitic infections (McKenzie, 1996). These cells have IgE antibody receptors. When these cells are activated degranulation occurs and histamine and leukotriene D4 are released resulting in vasodilatation, muscus secretion and smooth muscle contraction (Gorden-Smith, 2009b).
Erythrocytes or red blood cells have a life span of approximately 120 days. The actual number of circulating red blood cells varies depending on age, sex and location of the individual. Low red cell counts result in anemia which may give rise to tissue hypoxia. Haemoglobin is a protein tetramer within the red blood cell that can bind up to four molecules of oxygen. The binding affinity of haemoglobin is physiologically adjustable by a number of variables such as temperature, pH, the oxygen pressure (pO₂) and the given CO₂ pressure (pCO₂). Senescent erythrocytes are phagocytosed by the macrophages of the spleen, liver and marrow. Iron from the haem group is conserved and reused in erythropoiesis after binding to
transferrin and transported to the marrow. The porphyrin ring of the haem portion is catabolised to bilirubin by hepatocytes and excreted by the intestinal tract. Intravascular erythrocyte destruction results in the dissociation of haemoglobin into α and β subunits which bind to haptoglobin which transports the dimers to the liver where they are catabolised to bilirubin (Mckenzie, 1996; Gordon-Smith, 2009).

1.8 Platelets and haemostasis.

Platelets are produced in the bone marrow by the fragmentation of the cytoplasm of megakaryocytes. The normal platelet count varies from around approximately 150x 10⁹ / L to 400 x 10⁹ / L. The platelet functions to form plugs in response to vascular injury. Platelets exposed to collagen from compromised vascular endothelial cells result in the release of platelet granules and arachidonate resulting in the formation of thromboxane A2 which has vasoconstrictive properties. Platelet-derived growth factor found in platelet granules stimulates smooth muscle cells to replicate resulting in faster vascular healing. Tissue factor and platelet phospholipids activate the coagulation cascade which engenders fibrin to create a stable haemostatic plug (Figure 1.14 and 1.15). Platelet counts are determined in full blood counts and the efficiency of the coagulation cascade can be assessed by measuring the prothrombin time (PT) and activated partial thromboplastin time (APTT) (Hoffbrand & Pettit, 1999).
Figure 1.14. Coagulation pathway (Adapted from Hoffbrand & Pettit, 1999).

Figure 1.15. Haemostatis overview (Adapted from Hoffbrand & Pettit, 1999).
Biochemistry

2.1 Acute phase reactants / proteins.

Acute phase reactants (APR) are important molecules initiated by infection, inflammation and malignancy. APR induce cytokines such as (IL-1 and IL-6 type cytokines) and hormones such as insulin and synthesised by hepatocytes, monocytes, endothelial cells, fibroblasts and adipocytes. The primary role of APR is to function in haemostatic, antithrombotic, phagocytic, anti-proteolytic and antimicrobial processes. A number of important (but not all inclusive list) of APR and their functions are presented in Table 1.2 (Steel & Whitehead, 1994; Ebersole & Cappelli, 2000).

2.2 Renal Function.

The kidneys play pivotal roles in the formation and excretion of urine, homeostasis and endocrine function. Each kidney consists of hundreds of thousands of nephrons. Each nephron consists of a glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct. The proximal tubule reabsorbs 70-75 % of the sodium, chloride, bicarbonate, glucose, amino acids and water of the kidney filtrate. The proximal tubule secretes 90 % of the hydrogen ions excreted by the kidney. In the loop of Henle, there is a further 20-25% re-absorption of sodium. In regards to magnesium re-absorption, the thick ascending limb of the loop of Henle is responsible for 65-75% re-absorption of filtered magnesium (Swaminathan, 2003).
Table 1.2. Acute phase proteins / reactants (Adapted from Ebersole & Cappelli, 2000).

<table>
<thead>
<tr>
<th>Acute Phase Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>Opsonin and induction of IL-1ra</td>
</tr>
<tr>
<td>Serum amyloid A protein</td>
<td>Apoliprotein and chemoattractant</td>
</tr>
<tr>
<td>α 1-antitrypsin</td>
<td>Antiproteinase and induction of IL-1ra</td>
</tr>
<tr>
<td>α 1-acid glycoprotein</td>
<td>Transport and antiinflammatory properties</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Coagulation</td>
</tr>
<tr>
<td>α 1-proteinase inhibitor</td>
<td>Antiproteinase</td>
</tr>
<tr>
<td>α 1-antichymotrypsin</td>
<td>Antiproteinase</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Binds and removes haemoglobin</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>O₂ scavenger and transport</td>
</tr>
<tr>
<td>Complement C3</td>
<td>Opsonin</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transport</td>
</tr>
<tr>
<td>α 2-macroglobin</td>
<td>Antiproteinase</td>
</tr>
<tr>
<td>Transferrin (decreased)</td>
<td>Transport</td>
</tr>
</tbody>
</table>

In the distal tubule, there is a high degree of plasma electrolyte and acid/base regulation. The mechanism underpinning this regulation involves the exchange of hydrogen ions for sodium ions as well as potassium ion excretion. Serum urea,
creatinine and electrolytes provide the physician with important information in regards to homeostasis (Newman & Price, 2001).

2.2 Liver Function.

Hepatocytes transform water-insoluble bilirubin, the breakdown product of haeme produced by the reticuloendothelial system, by coupling to glucuronic acid to produce water-soluble bilirubin mono- and di-glucurides. These compounds are then excreted into the bile. The liver is a major producer of coagulation factors, APR (discussed above) and bile acids. The liver catabolises a number of hormones such as thyroid and steroid hormones and plays a pivotal role in drug metabolism. Decreased liver function may occur due to apoptosis and necrosis of hepatocytes. In the laboratory liver function tests include measuring serum bilirubin (conjugated and total), the concentration of serum enzymes such as alanine transaminase (ALT), aspartate transaminase AST, GGT and alkaline phosphatase (AP) and serum albumin (Tolman & Rej, 2001).
3.

Overview and Aims of this study

Patients suffering from leptospirosis often present with symptoms similar to those of other tropical diseases such as fever, headache, joint pain, rigours, making differential diagnosis difficult. Moreover, there is still much work to be done in clarifying the pathogenesis of leptospirosis. Thus, the over-arching theme of the work reported in this thesis is an examination of haematological and biochemical markers in the blood of patients with leptospirosis which may help to point to improvements in diagnoses and further an understanding of pathogenesis. The specific aims of this study are discussed in more detail below.

Some reports in the literature suggest possible markers which may be used in differential diagnoses. In a clinical setting, a retrospective review of 34 patients with leptospirosis admitted to Pontchaillou Hospital located in metropolitan France, observed that 85% of leptospirosis patients were lymphopenic and concluded that lymphopenia is a common feature of leptospirosis (Jauréguiberry et al., 2005). In response to this finding, Lopes, Costa and Sacramento (2005) reviewed 253 leptospirosis patients in Salvador, Brazil and conversely observed that only 17% of patients were lymphopenic at admission. The reports seem contradictory, although little analysis in either study was directed towards whether the infecting serovar contributed to these apparently conflicting results. Thus, the first aim of this study
is to investigate whether lymphopenia (if it does occur in leptospirosis) is serovar-dependant.

Neither of the aforementioned studies (Jauréguiberry et al., 2005, Lopes, Costa and Sacramento, 2005) analysed which phase (acute or immune) of the disease their patients were in. Thus, the second aim of this study is to investigate whether lymphopenia depends on whether the patient is in the acute or immune phase.

In patients with leptospirosis and with acute lung injury, haemodynamic disturbance, or serum potassium level > 4 mmol/L, or a serum creatinine level > 265.2 μmol/L has been associated with mortality (Marotto et al., 1999). Similarly, Esen et al. (2004) compared the laboratory test results of 72 patients diagnosed with leptospirosis and found significant elevations in serum potassium, aspartate transaminase (AST), alanine transaminase (ALT) and prothrombin time prolongation when comparing survivors to non-survivors. While both these studies addressed laboratory makers associated with mortality, neither study investigated laboratory markers associated with patients with severe disease who required admission to an intensive care unit and survived. Therefore the third aim of this study is to determine if differences in laboratory markers exist to differentiate those with an uncomplicated mild episode of leptospirosis and those who were admitted to an intensive care unit with severe disease.

Magnesium is the fourth-most abundant cation in the body and plays a diverse role in the physiology of many organ systems in the body (Swaminathan
Studies that have reported on the serum magnesium status of patients with leptospirosis have mixed findings. Early reports from Brazil revealed that leptospirosis patients had higher serum magnesium levels than healthy controls (de Jorge, 1970). Conversely, a recent report from Thailand revealed hypomagnesemia in 50% of leptospirosis patients and a case study reported on an acute leptospirosis patient who displayed severe hypomagnesemia and required large doses of magnesium replacement during the acute phase (Khositseth et al., 2008; Spichler et al., 2008). The paradox engendered by the juxtaposition of these studies suggest that more data is required to determine if serum magnesium status is dramatically altered in leptospirosis. **Thus the fourth aim of this study is to evaluate the magnesium status in patients during the acute phase (first 10 days of infection) to determine if they develop hypomagnesemia.**

Whilst there have been reports of simultaneous infection with more than one serovar (Doherty, 1958), reports on host responses to consecutive episodes of leptospirosis have eluded the research literature. **Thus, the fifth aim of this study is to determine if laboratory markers, in a second consecutive infection with a different serovar of leptospires, are markedly different.**

Associations between auto-immune antibodies or auto-immune disease and leptospirosis have been reported several times. Anti-neutrophil cytoplasmic antibodies (Constantin et al., 1996), anti-cardiolipin antibodies (Daher et al., 2002), anti-phospholipid syndrome (Tattevin et al., 2003) and reactive arthritis (Pappas et al., 2003), for example, have all been associated with leptospirosis. Of the cases of
leptospirosis investigated by Santiago et al. (2001), 23%, 10% and 17% carried anti-cardiolipin IgG, anti-cardiolipin IgM and anti-2-glycoprotein-I auto-antibodies, respectively. Antibodies directed toward the leptospiral LruA and LruB lipoproteins have been implicated in the auto-immune aspects of leptospiral uveitis (Verma et al., 2005), and sera from most (>65%) of the Indian cases of leptospiral uveitis recently investigated by Verma et al. (2008) were found to contain antibodies to these lipoproteins. Interestingly, Barnabe and Fahlman (2008) have delineated several clinical features that are shared by lupus and leptospirosis.

Goodpasture’s syndrome, a severe auto-immune disease causing glomerulonephritis, with or without pulmonary haemorrhage, is rapidly fatal without aggressive medical intervention. Since leptospirosis may facilitate glomerulonephritis and pulmonary dysfunction it is surprising that leptospirosis has not been considered in the development of Goodpasture’s syndrome. Therefore the sixth aim of this study is to determine if leptospirosis patients produce auto antibodies to type 4 collagen (the Goodpasture antigen).
Chapter 2

The influence of serovars on lymphopenia in patients with leptospirosis.
This chapter resulted in the following publication


2.1 INTRODUCTION

The main aim of the work reported in this thesis was to investigate host responses in leptospirosis. To facilitate this aim, haematological and clinical biochemical markers were investigated in patients with leptospirosis. During the course of this work, it became apparent that one possible marker for leptospirosis is lymphopenia. As discussed in Chapter 1, lymphocytes (Figure 1.7) are the second most abundant leukocyte circulating in the peripheral blood. The normal number varies between 1.2 and \(4 \times 10^9\) / L. Lymphocytosis is the term used to describe the condition when the number of lymphocytes seen in a peripheral blood smear is \(> 4 \times 10^9\) / L. Lymphopenia is observed when the number of lymphocytes seen in a peripheral blood smear is \(< 1.5 \times 10^9\) / L. (Carrick & Begg, 2008; McKenzie, 1996). Lymphocytes as a group of leukocytes are divided into two groups: T and B lymphocytes. Lymphocytes are pivotal in the production of immunoregulatory cytokines and underpin host humoral and cell mediated immune responses.

Anti-lymphoid activity by leptospiral exoproducts was first observed by Oravec and Kmety (1978). Oravec and Kmety reported that following the intraperitoneal application of supernatant fluid of \(L.\) biflexa serovar Patoc 1 in Syrian hamsters there was a pronounced and transient fall in the hamster’s peripheral lymphocyte counts. In a clinical setting, a retrospective review of 34 patients with leptospirosis admitted to Pontchaillou Hospital located in metropolitan France, observed that 85% of leptospirosis patients were lymphopenic and concluded that lymphopenia is a common feature of leptospirosis (Jauréguiberry et al., 2005). In
response to this finding, Lopes, Costa and Sacramento (2005) reviewed 253 leptospirosis patients in Salvador, Brazil and conversely observed that only 17 % of patients were lymphopenic at admission. Lopes, Costa and Sacramento (2005) suggested that environmental factors and the different distribution of leptospiral serovars may account for the differences observed in the frequency of lymphopenia. The most common serovar in Salvador, Brazil is \textit{L. interrogans} serovar Copenhageni (Tucunduva de Faria \textit{et al.}, 2008; Ko \textit{et al.}, 1999) while in the Pontchaillou Hospital study the most common serovar was Grippotyphosa (species \textit{L. interrogans} or \textit{L. Kischneri}) (Jauréguiberry \textit{et al.}, 2005). Both studies failed to report the frequency of lymphopenia across serovars.

In response to the contradictory findings of these two studies in relation to lymphopenia, and the suggestion that lymphopenia may, at least in part, be serovar dependant, the incidence of lymphopenia ($< 1.2 \times 10^9$ lymphocytes / L) in 258 patients diagnosed with leptospirosis at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis in Queensland, Australia was investigated to determine if the frequency of lymphopenia is serovar-dependant.
2.2 PATIENTS AND METHODS

This study was approved by the Human Ethics Committee from Queensland Health Forensic and Scientific Services (08-001/12) and the Human Ethics Research Committee from the University of the Sunshine Coast (A/08/155). A total of 258 cases of leptospirosis were identified and investigated retrospectively using the leptospirosis patient database at the WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis. Leptospirosis was confirmed when leptospires, were isolated from blood cultures in Ellinghausen-McCullough-Johnson-Harris (EMJH) media or detected by real time PCR. Leptospirosis was also confirmed if there was a greater than or equal to 4 fold rise in microscopic agglutination test titre on follow up samples following initial presentation or a MAT titre $\geq 400$. Other diseases causing pyrexia of unknown origin were excluded by serological testing and blood cultures. Pathology results interrogated were those from the first samples taken following presentation at a Queensland Health hospital.
2.3 RESULTS AND DISCUSSION

The lymphocyte counts for the 258 patients reviewed are presented in Table 2.1. Of these 258 patients, 238 (92%) were lymphopenic. This result is consistent with those observed in the studies of Jauréguiberry et al. (2005) and recently Abgueguen et al. (2008), which reported lymphopenia in 85% and 63% of their leptospirosis patients respectively. *Prima facie*, these findings are inconsistent with those of Lopes, Costa and Sacramento (2005), who observed lymphopenia in only 17% of their leptospirosis patients. However, these authors point out, based on other data (see Ko et al., 1999 and Tucunduva de Faria et al., 2008) that *L. interrogans* serovar Copenhageni was the most frequent serovar isolated from their study. Examining the literature on which this statement is based, it is estimated that the frequency of *L. interrogans* serovar Copenhageni in Salvador, Brazil to be as high as 90%. This contrasts markedly with the results of this study and that of Jauréguiberry et al. (2005), where the frequency of *L. interrogans* serovar Copenhageni is 2% and 12% respectively. It is noted that the identification of *L. interrogans* serovar Copenhageni in the study of Jauréguiberry et al. (2005) was presumptive only. The results of this chapter show a high incidence of lymphopenia in patients infected with most serovars: for 15 of the 18 identified serovars, more than 90% of patients were lymphopenic. The three exceptions were *L. borgpetersenii* serovar Arborea 81% of patients were lymphopenic, N=16), *L. borgpetersenii* serovar Hardjo (72%, N=18) and *L. interrogans* serovar Copenhageni (40%, N=5). Whilst the incidence of lymphopenia observed with *L. interrogans* serovar Copenhageni in this study is higher that that seen in the study of Lopes,
Costa and Sacramento (2005) (17%, N=253), the *L. interrogans* serovar Copenhageni sample size in this study was small. Nevertheless, it is striking that patients infected with this serovar exhibited the lowest incidence of lymphopenia in the study.

Lymphopenia during the acute phase of infection appears common across the majority of pathogenic serovars routinely screened for at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis. Previously, even *L. biflexa* Serovar Patoc 1, a non pathogenic serovar has been shown to induce lymphopenia (Oravec & Kmety 1978). In concluding, it is submitted that lymphopenia is a common finding in leptospirosis across serovars with *L. interrogans* serovar Copenhageni a possible exception. This chapter is in agreement with Lopes, Costa and Sacramento (2005) that the frequency of lymphopenia in leptospirosis patients may vary across regions and factors that may cause variations in patient lymphopenia are worthy of future research as are the mechanisms underpinning the phenomena. The latter may provide valuable insights into the future treatments of leukemic conditions.
Table 2.1. Number of lymphopenic patients as a function of leptospiral Serovar.

<table>
<thead>
<tr>
<th>Leptospira Serovar</th>
<th>n</th>
<th>Mean Lymphocyte Count (x 10^9/L)</th>
<th>Number of Lymphopenic Patients (&lt; 1.2 x 10^9/L)</th>
<th>% Lymphopenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. borgpetersenii serovar Arborea</td>
<td>16</td>
<td>0.79</td>
<td>13</td>
<td>81.3</td>
</tr>
<tr>
<td>L. interrogans serovar Australis</td>
<td>47</td>
<td>0.62</td>
<td>44</td>
<td>93.6</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Ballum</td>
<td>6</td>
<td>0.54</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Bendjei</td>
<td>1</td>
<td>0.46</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>L. kirschneri serovar Bulgareca</td>
<td>4</td>
<td>0.70</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Canicola</td>
<td>7</td>
<td>0.86</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>L. weilii serovar Celledoni</td>
<td>6</td>
<td>0.69</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Copenhageni</td>
<td>5</td>
<td>1.38</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Hardjo*</td>
<td>18</td>
<td>0.86</td>
<td>13</td>
<td>72.2</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Javanica</td>
<td>1</td>
<td>0.62</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Kremastos or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. santarosai serovar Kremastos</td>
<td>16</td>
<td>0.71</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Medanensis</td>
<td>2</td>
<td>0.46</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Pomona or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. noguchii serovar Pomona</td>
<td>5</td>
<td>0.60</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Robinsoni</td>
<td>18</td>
<td>0.69</td>
<td>17</td>
<td>94.4</td>
</tr>
<tr>
<td>L. interrogans serovar Szwajzak or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. santarosai serovar Szwajzak</td>
<td>8</td>
<td>0.54</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Tarassovi</td>
<td>10</td>
<td>0.30</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>L. weilii serovar Topaz</td>
<td>9</td>
<td>0.46</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>L. interrogans serovar Zanoni</td>
<td>77</td>
<td>0.67</td>
<td>73</td>
<td>94.8</td>
</tr>
<tr>
<td>Isolate not recovered</td>
<td>2</td>
<td>0.88</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>258</td>
<td><strong>0.68</strong></td>
<td><strong>238</strong></td>
<td></td>
</tr>
</tbody>
</table>

* L. interrogans serovar Hardjo or L. meyeri serovar Hardjo have not been demonstrated in Australia.
Chapter 3

The influence of the phase of infection on lymphopenia in patients with leptospirosis
This chapter resulted in the following publication

3.1 INTRODUCTION

The results from the investigation undertaken in Chapter 2 show a high incidence of lymphopenia in patients infected with most serovars: for 15 of the 18 identified serovars in reported in Chapter 2, more than 90% of patients were lymphopenic. Lymphopenia during leptospirosis appears common across the majority of pathogenic serovars routinely screened for at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis, and is consistent with anti-lymphoid activity by leptospiral exoproducts as first observed by Oravec and Kmety (1978).

As previously discussed in Chapter 2, there are two contradictory reports in the literature: that of Jauréguiberry et al. (2005) concerning Pontchaillou Hospital located in metropolitan France and showing that 85% of leptospirosis patients were lymphopenic and that of Lopes, Costa & Sacramento, 2005 reporting that only 17% of patients were lymphopenic at admission. The results in Chapter 2 offer some clarification, indicating that lymphopenia is partly due to the nature of the serovar. Nevertheless, other factors may also contribute to these apparently contradictory results. Unfortunately, both studies failed to analyse their lymphocyte count data in relation to whether their patients were in the leptospiremic or immune phase of the disease. In response to this stark contrast in lymphocyte counts between the two previous studies, this study investigated the incidence of lymphopenia (< 1.2 x 10⁹ lymphocytes / L) in 57 patients diagnosed with leptospirosis at the WHO/FOA/OIE Collaborating Centre for Reference and Research on Leptospirosis in Queensland,
Australia to determine if lymphocyte counts from the acute phase or immune phase vary in leptospirosis.

3.2 PATIENTS AND METHODS

A total of 57 cases of leptospirosis were identified and investigated retrospectively using the leptospirosis patient database at the WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis. Leptospirosis was confirmed when leptospires were isolated from blood cultures in Ellinghausen-McCullough-Johnson-Harris (EMJH) media or detected by real time PCR. Leptospirosis was also confirmed if there was a greater than or equal to 4 fold rise in microscopic agglutination test titre on follow up samples following initial presentation or a microscopic agglutination test (MAT) titre ≥ 400. To determine if serological status affects the lymphocyte counts in leptospirosis patients the study determined the serostatus of all 57 patients at their initial lymphocyte count following presentation at a Queensland Health hospital. Thirty-three patients were *Leptospira* IgM non reactive by ELISA, 10 were *Leptospira* IgM reactive by ELISA and non reactive by MAT and 14 were both *Leptospira* IgM reactive by ELISA and reactive by MAT. Other diseases causing pyrexia of unknown origin had been excluded by serological testing and blood cultures. Lymphocyte counts from the three groups were compared using the t-Test statistical function in Microsoft Excel. Lymphocyte counts were considered significant for p values < 0.05.
3.3 RESULTS AND DISCUSSION

The mean lymphocyte counts for the three groups are summarised in Table 3.1. The lymphocyte counts summarised in Table 3.1 reveal that there was no significant difference in lymphocyte counts between patients Leptospira IgM non reactive and patients Leptospira IgM reactive and MAT non reactive (0.55 x 10^9 / L versus 0.65 x 10^9 / L, \( p = 0.45 \)). There was a significant difference observed in lymphocyte counts between patients Leptospira IgM non reactive and patients Leptospira IgM reactive and MAT reactive (0.55 x 10^9 / L versus 1.57 x 10^9 / L, \( p < 0.001 \)). A significant difference was also observed in lymphocyte counts between patients Leptospira IgM reactive and MAT non reactive and patients Leptospira IgM reactive and MAT reactive (0.65 x 10^9 / L versus 1.57 x 10^9 / L, \( p = 0.001 \)).

Lymphocyte counts in patients first presenting with leptospirosis have proven variable. Reports on the frequency of lymphopenia in leptospirosis patients have been as high as 85 % and as low as 17 % (Jauréguiberry et al., 2005; Lopes, Costa & Sacramento, 2005). The results of this study are consistent with previous studies showing a high incidence of lymphopenia in leptospirosis. During the acute phase when patients are IgM negative and leptospires are in circulation excreting lymphotoxic exoproducts (Oravec & Kmety, 1978), lymphopenia was observed in 100 % of leptospirosis patients. Similarly, results in this study suggest that when patients were IgM reactive and MAT non-reactive (at the end of the acute phase and beginning of the immune phase, when lymphotoxic exoproducts may still be circulating) 90% of leptospirosis patients are lymphopenic. During the immune phase when patients are IgM reactive and MAT reactive a lower frequency of
lymphopenia (50%) is observed. Further, three of the seven lymphopenic patients (43%) in the immune phase (IgM reactive and MAT reactive) had only a mild lymphopenia (1.14, 1.17 and 1.17 x 10^9 / L respectively). This is possibly due to immunological clearance of leptospires and their lymphotoxic exoproducts from circulation (Faine et al., 1999).

In conclusion this study has shown that the phase of the leptospiral infection may affect the initial observed lymphocyte count and explain some of the variation in lymphocyte counts previously reported in leptospirosis. Further, the lymphocyte count observed in patients (particularly during the acute phase) will guide the clinician to the most appropriate test (culture or PCR verses MAT) to confirm or exclude the diagnosis. This is significant as the MAT is inappropriate during the acute phase and culture or PCR is inappropriate during the immune phase. Moreover, determining which phase of the disease the patient is in may assist the clinician to determine the utility of the administration of antibiotics.
Table 3.1. Lymphocyte counts as a function of serological status in leptospirosis patients at initial presentation.

<table>
<thead>
<tr>
<th>Serological status</th>
<th>n</th>
<th>% lymphopenic</th>
<th>Mean lymphocyte count (x 10^9 / L)</th>
<th>Standard error</th>
<th>Comparison</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(&lt;1.2 x 10^9 / L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. IgM Non Reactive</td>
<td>33</td>
<td>100</td>
<td>0.55</td>
<td>0.04</td>
<td>1 vs 2</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 vs 3</td>
<td>&gt; 0.001</td>
</tr>
<tr>
<td>2. IgM Reactive + MAT Non Reactive</td>
<td>10</td>
<td>90</td>
<td>0.65</td>
<td>0.12</td>
<td>2 vs 3</td>
<td>0.001</td>
</tr>
<tr>
<td>3. IgM Reactive+ MAT Reactive</td>
<td>14</td>
<td>50</td>
<td>1.57</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4

Haematological and clinical chemistry markers in patients with uncomplicated leptospirosis and patients with severe leptospirosis: A comparison of laboratory findings upon initial presentation.
This chapter resulted in the following publication

4.1 INTRODUCTION

In the course of analysing the data presented in the previous two chapters, it became apparent that an important avenue of enquiry would be to determine if haematological and biochemical markers differ in those with either a mild, uncomplicated episode of leptospirosis or a severe episode of leptospirosis.

The more severe form of leptospirosis involves multi organ failure. Some of the clinical features of the severe form of leptospirosis include renal failure, liver failure, myocarditis and pulmonary dysfunction (Faine, et al., 1999). Pulmonary manifestations in leptospirosis have been estimated to occur in 20% to 70% of cases (O’Neil, et al., 1991). The mild, benign forms of the pulmonary involvement are evidenced by haemoptysis, while the more life threatening form of pulmonary involvement is evidenced by pulmonary haemorrhage in the form of focal or diffuse areas of alveoli imbued with erythrocytes (Carvalho & Bethlem, 2002). While a poor prognosis is associated with ≥ 10 000 Leptospires / mL of blood or milligram of tissue, it remains unclear as to whether the pulmonary manifestations are due to a leptosiral toxin, an exacerbated host immune response, or a combination of both (Segura et al., 2005; Dolhnikoff, et al., 2007).

In leptospirosis patients with acute lung injury, haemodynamic disturbance, or serum potassium level > 4 mmol/L or a serum creatinine level > 265.2 μmol/L has been associated with mortality (Marotto et al., 1999). Similarly, Esen et al. (2004) compared the laboratory test results of 72 patients diagnosed with leptospirosis and
found significant elevations in serum potassium, aspartate transaminase (AST), alanine transaminase (ALT) and prothrombin time prolongation when comparing survivors to non-survivors.

The aim of this study was to retrospectively compare laboratory findings from the first blood samples taken following hospital presentation in those with uncomplicated leptospirosis and those admitted to a high dependency medical ward or ICU with severe leptospirosis in an attempt to identify laboratory markers that differentiate both groups.
4.2 PATIENTS AND METHODS

The study protocol was approved by the Human Ethics Committee from Queensland Health Forensic and Scientific Service (Approval Number 08-001/12) and the Human Ethics Research Committee from the University of the Sunshine Coast (Approval Number A/08/155). A total of 239 leptospirosis patients between the ages of 18 and 75 years were identified and investigated retrospectively over a 10 year period using the leptospirosis patient database at the WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis. Of the 239 cases investigated, 12 patients, all male, displayed significant respiratory distress as evidenced by dyspnea, haemoptysis, diffuse alveolar haemorrhage and or acute liver or renal failure were admitted to a high dependency (HDU) or intensive care unit (ICU). Following an average stay of 12 days in the HDU or ICU all 12 patients eventually recovered from their infection. The remaining 227 patients, also all male, did not require hospitalization in a HDU or ICU and recovered from an unremarkable episode of leptospirosis. Leptospirosis was confirmed when leptospires were isolated from blood cultures in Ellinghausen-McCullough-Johnson-Harris (EMJH) media or detected in serum by real time PCR. Leptospirosis was also confirmed serologically if there was a greater than, or equal to, 4 fold rises in microscopic agglutination titre on follow up from the initial presentation, or an MAT titre ≥ 400. Common diseases causing pyrexia in Australia were excluded by serological testing and blood cultures. For example infections with Dengue virus, Ross River virus, Barmah Forest virus, Ricketsia spp. (Spotted Fever) and Orientia spp. (Scrub Typhus) were excluded by serology. Negative blood cultures excluded infections with pathogenic
Staphylococcus ssp., Streptococcus ssp., Menigococcus ssp., Pseudomonas ssp., Haemophilus ssp., and obligate anaerobic bacteria. Pathology results were those from the first samples taken following initial presentation at a Queensland Health hospital. Laboratory findings of both groups were compared using the t-Test statistical function in Microsoft Excel. Haematological and biochemical markers were considered significant for p values < 0.05.
4.3 RESULTS

The laboratory results for both groups are presented in Tables 1 and 2. The haematological laboratory results presented in Table 4.1 reveal that the patients with severe leptospirosis had a significantly lower mean haemoglobin (122.3 g/L versus 145.3 g/L, \( p = 0.005 \)), platelet count (109.8 x 10^9/L versus 162.4 x 10^9/L, \( p = 0.03 \)), haematocrit (0.36 versus 0.43; \( p = 0.003 \)) and red cell count (4.1 x 10^{12}/L versus 4.8 x 10^{12}/L, \( p = 0.01 \)). These patients also presented with a significantly higher mean white cell count (13.7 x 10^9/L versus 8.9 x 10^9/L, \( p = 0.007 \)) and neutrophil count (11.9 x 10^9/L versus 7.8 x 10^9/L, \( p = 0.009 \)). There was no significant difference in the lymphocyte count between the groups (0.80 x 10^9/L versus 0.66 x 10^9/L, \( p = 0.3 \)).

There was no data to compare both groups in terms of prothrombin time (PT), activated partial thromboplastin time (APTT) or fibrin. Eleven of the 12 patients with severe leptospirosis had their PT measured. Of these, six had a PT above the upper limit of the reference range (> 12 seconds). Further, eight of the 12 patients with severe leptospirosis had their fibrin measured. Of these, all eight had a fibrin level above the upper limit of the reference range (4.5 g/L). Eleven of the 12 patients with severe leptospirosis had their APTT measured. Of these, two had an APTT above the upper limit of the reference range (> 39 seconds).

The biochemistry laboratory results presented in Table 4.2 reveal that the patients with severe leptospirosis had a significantly lower mean serum; protein (63.9 g/L versus 71.8 g/L, \( p = 0.007 \)) and albumin (32.4 g/L versus 40.4 g/L, \( p = 0.002 \)).
Table 4.1. Differences in haematology test results from two groups of leptospirosis patients.

<table>
<thead>
<tr>
<th>Haematological Markers</th>
<th>Normal Reference Range</th>
<th>Uncomplicated leptospirosis Mean</th>
<th>% of 227 patients with data</th>
<th>Severe Leptospirosis Mean</th>
<th>% of 12 patients with data</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/L)</td>
<td>135-185</td>
<td>145.3 0.9</td>
<td>98.2</td>
<td>122.3 6.6</td>
<td>100 0.005</td>
<td></td>
</tr>
<tr>
<td>White cell count (x 10^9/L)</td>
<td>4-11</td>
<td>8.9 0.3</td>
<td>98.2</td>
<td>13.7 1.7</td>
<td>100 0.007</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x 10^9/L)</td>
<td>2-8</td>
<td>7.8 0.3</td>
<td>98.2</td>
<td>11.9 1.5</td>
<td>100 0.009</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x 10^9/L)</td>
<td>1.2-4</td>
<td>0.66 0.02</td>
<td>98.2</td>
<td>0.80 0.1</td>
<td>100 0.30</td>
<td></td>
</tr>
<tr>
<td>Platelets (x 10^9/L)</td>
<td>140-400</td>
<td>162.4 3.8</td>
<td>96.9</td>
<td>109.8 20.2</td>
<td>100 0.03</td>
<td></td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.39-0.52</td>
<td>0.43 0.003</td>
<td>98.2</td>
<td>0.36 0.02</td>
<td>100 0.003</td>
<td></td>
</tr>
<tr>
<td>Red cell count (x 10^12/L)</td>
<td>4.5-6</td>
<td>4.8 0.03</td>
<td>98.2</td>
<td>4.1 0.2</td>
<td>100 0.01</td>
<td></td>
</tr>
<tr>
<td>Prothrombin time (seconds)</td>
<td>9-12</td>
<td>- -</td>
<td>0</td>
<td>13.2 0.6</td>
<td>91.7 -</td>
<td></td>
</tr>
<tr>
<td>Activated partial thromboplastin time (seconds)</td>
<td>24-39</td>
<td>- -</td>
<td>0</td>
<td>35.7 2.0</td>
<td>91.7 -</td>
<td></td>
</tr>
<tr>
<td>Fibrin (g/L)</td>
<td>1.7-4.5</td>
<td>- -</td>
<td>0</td>
<td>7.3 0.6</td>
<td>66.7 -</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2. Differences in biochemical test results from two groups of leptospirosis patients.

<table>
<thead>
<tr>
<th>Biochemistry Markers</th>
<th>Normal Reference Range</th>
<th>Uncomplicated leptospirosis</th>
<th>% of 227 patients with data</th>
<th>Severe Leptospirosis</th>
<th>% of 12 patients with data</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mmol/L)</td>
<td>135-145</td>
<td>134.4</td>
<td>0.24</td>
<td>99.6</td>
<td>132.8</td>
<td>1.67</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>3.5-4.5</td>
<td>3.8</td>
<td>0.04</td>
<td>97.8</td>
<td>3.6</td>
<td>0.14</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>100-110</td>
<td>98</td>
<td>0.30</td>
<td>96.5</td>
<td>97.3</td>
<td>2.13</td>
</tr>
<tr>
<td>Anion Gap (mmol/L)</td>
<td>4-13</td>
<td>12</td>
<td>0.24</td>
<td>96</td>
<td>13.3</td>
<td>1.06</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>2.1-7.1</td>
<td>6.4</td>
<td>0.28</td>
<td>99.6</td>
<td>12.5</td>
<td>1.73</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>70-120</td>
<td>117</td>
<td>5.04</td>
<td>99.6</td>
<td>211.3</td>
<td>33.72</td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>60-83</td>
<td>71.8</td>
<td>0.46</td>
<td>100</td>
<td>63.9</td>
<td>2.43</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35-50</td>
<td>40.4</td>
<td>0.39</td>
<td>100</td>
<td>32.4</td>
<td>2.00</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>25-45</td>
<td>31.5</td>
<td>0.29</td>
<td>100</td>
<td>31.4</td>
<td>1.01</td>
</tr>
<tr>
<td>Bilirubin (Total - µmol/L)</td>
<td>&lt; 20</td>
<td>17</td>
<td>0.73</td>
<td>99.6</td>
<td>25.1</td>
<td>4.29</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>53-128</td>
<td>105.7</td>
<td>4.84</td>
<td>100</td>
<td>84.8</td>
<td>9.22</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase (U/L)</td>
<td>&lt; 55</td>
<td>72.9</td>
<td>6.19</td>
<td>100</td>
<td>88.1</td>
<td>30.71</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>&lt; 45</td>
<td>56.2</td>
<td>4.79</td>
<td>100</td>
<td>40.4</td>
<td>6.81</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>&lt; 35</td>
<td>59.6</td>
<td>4.91</td>
<td>99.1</td>
<td>77.3</td>
<td>16.64</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>110-250</td>
<td>296.4</td>
<td>23.11</td>
<td>7.6</td>
<td>275.3</td>
<td>118.27</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>0.81-1.45</td>
<td>1</td>
<td>0.08</td>
<td>12.3</td>
<td>0.9</td>
<td>0.08</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>&lt; 5</td>
<td>186.9</td>
<td>18.55</td>
<td>14.1</td>
<td>217.5</td>
<td>55.41</td>
</tr>
<tr>
<td>pH</td>
<td>7.35-7.45</td>
<td>-</td>
<td>0</td>
<td>7.14</td>
<td>0.04</td>
<td>33.3</td>
</tr>
<tr>
<td>pCO₂</td>
<td>35-45</td>
<td>-</td>
<td>0</td>
<td>48.8</td>
<td>7.85</td>
<td>33.3</td>
</tr>
<tr>
<td>pO₂</td>
<td>75-100</td>
<td>-</td>
<td>0</td>
<td>76.7</td>
<td>8.79</td>
<td>33.3</td>
</tr>
</tbody>
</table>
These patients also presented with a significantly higher mean urea (12.5 mmol/L versus 6.4 mmol/L, \( p = 0.004 \)) and creatinine (211.3 µmol/L versus 117 µmol/L, \( p = 0.02 \)). There was no data to compare both groups in terms of blood gases such as pH, pCO\(_2\) or pO\(_2\). Four of the 12 patients with severe leptospirosis had their blood gases measured. Of these, all four had a blood pH level below the lower limit of the reference range (7.35). Further, three of the same four patients had a pCO\(_2\) above the upper limit of the reference range (45 mmHg) and two of the four patients with measured blood gases had a pO\(_2\) below the lower limit of the reference range (75 mmHg).
4.4 DISCUSSION

The aim of this study was to compare and identify differences between the initial laboratory findings of two groups of leptospirosis patients: those with severe leptospirosis admitted to a high dependency ward or intensive care unit and those who recovered from an uncomplicated episode of leptospirosis.

The significantly lower haemoglobin, red cell count and haematocrit in the severe leptospirosis group is possibly due to compromises in the integrity of the vascular system, as a result of the lower platelet count also observed in the severe disease group. It was recently shown that thrombocytopenia was the only haemostatic factor independently associated with bleeding and it should be considered as a predictor of the severity of leptospirosis (Chierakul et al., 2008). The cause of thrombocytopenia observed in the more severe cases of leptospirosis, whether host derived or from leptospires, or a combination of both, is yet to be elucidated, however it has been well established that platelet counts decrease due to their migration into the lungs and liver during endotoxemia (Stohlawetz et al., 1999). Immunohistochemical and ultrastructure studies suggest that thrombocytopenia is a result of platelet activation, adhesion and aggregation around the vascular endothelium (Nicodemo et al., 1997). The significantly lower haemoglobin, red cell count and haematocrit in severe leptospirosis may also be due to a toxic effect of leptospires on erythroid precursors in the bone marrow (Somers et al., 2003).
Leukocytosis and neutrophilia are common in leptospirosis (Kobayashi, 2001; Lee & Liu, 2007; Shah & Katira, 2007; Chierakul et al., 2008). While Marotto et al. (1999) and Esen et al. (2004) found no significant differences in leukocyte counts between survivors and non-survivors, this study observed significantly higher leukocyte and neutrophil counts between those with severe leptospirosis and those with uncomplicated leptospirosis. The difference between these findings and those of Marotto et al. (1999) and Esen et al. (2004) may be explained because all patients in this study survived. The study reported in this chapter is also more statistically robust given the larger sample size compared to the earlier studies. The observed neutrophilia in leptospirosis is consistent with the important role these cells play in the phagocytic uptake and intracellular destruction of microbial pathogens (Nathan, 2006). Research also suggests that, in septic patients, platelets aggregate and bind directly to neutrophils. This binding may facilitate the release of chromatin and azurophilic and gelatinase granules to form neutrophil extracellular traps (NETs) in the microvasculature where organisms can be trapped and destroyed (Brinkmann et al., 2004; Clark et al., 2007; Ma & Kubes 2008). The possible binding of platelets to neutrophils may also provide insights into the thrombocytopenia observed here and elsewhere.

Lymphopenia has recently been reported as a salient laboratory finding in leptospiral infections (Jauréguiberry et al., 2005; Craig et al., 2009). Other research has failed to support this observation (Lopes et al., 2005). In this study, although there was no significant difference in lymphocyte count between the two groups, both groups were clearly lymphopenic, a result consistent with previous research.
(Jauréguiberry et al., 2005; Craig et al., 2009) and an earlier postulation of anti-lymphoid activity in leptospiral exoproducts (Oravec and Kmety, 1978). The mechanisms underpinning the lymphopenia and anti-lymphoid activity remain to be elucidated. The prolonged prothrombin time and elevated fibrinogen in the severe leptospirosis group has been reported previously and is believed to be unrelated to the occurrence of a disseminated intravascular coagulation syndrome (Lomar et al., 2000).

Renal insufficiency in leptospirosis is commonly reported (Lombardi, 1997; McBride et al., 2005). The significantly elevated serum urea and creatinine observed in the severe leptospirosis group is consistent with Covic et al. (2003). Impaired proximal sodium reabsorption and increased distal sodium delivery and potassium wasting are characteristic of the renal insufficiency observed in leptospirosis (Abdulkader et al., 1996; McBride, et al., 2005). In addition to hypokalemia, hyperkalemia is also reported in leptospirosis, possibly due to rhabdomyolysis or more acute renal dysfunction (Sitprija, 2008). There was no significant difference in serum potassium levels between the two groups studied here. Given that all of the patients in this study survived, the non-significant difference in serum potassium is consistent with previous research suggesting an association of normokalemia and survival and hyperkalemia and mortality in leptospirosis (Marotto et al., 1999; Esen et al., 2005).

The significantly lower mean albumin in the severe leptospirosis group is of interest since it has been shown to be a protective factor against the toxic leptospiral
derived fatty acids that inhibit kidney sodium-potassium ATPase (Burth et al., 2005). The mechanism by which albumin exerts its protective effect on the sodium-potassium ATPase remains to be elucidated. This is also consistent with Sung et al. (2004) who found that a low level of albumin at admission is a significant predictor of mortality in trauma patients. Combined, these findings suggest the need to investigate intravenous albumin in the treatment of acute lung injury in leptospirosis.

Abnormal liver function tests are commonly reported in leptospirosis (Kobayashi, 2001; Jauréguiberry et al., 2005). The failure to find significantly higher levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the severe leptospirosis group is inconsistent with results of Esen et al. (2004) who found significant elevations in AST and ALT when comparing survivors to non-survivors. These inconsistent results are again possibly due to the fact that all of the patients in this study survived. Further, it should be noted that both the mean ALT and AST levels for both groups is higher than the upper limit of the normal reference range. Lee and Liu (2007) suggest that the high AST levels may also be due to AST derived from rhabdomyolysis and not just the liver because concurrent myalgia, myositis, hypermyoglobinemia are often found in leptospirosis.

In conclusion, when leptospirosis is suspected, patients presenting with any of the following; serum urea greater than 7.1 mmol/L, creatinine greater than 120 mmol/L, albumin less than 35 g/L, haemoglobin less than 135 g/L, haematocrit below 0.36, red cell count less than $4.5 \times 10^9$/L, platelet count less than $140 \times 10^9$/L, a
white cell count greater than $11 \times 10^9/L$ and neutrophil count greater than $8 \times 10^9/L$ may need to be closely monitored and have aggressive treatment regimes directed toward them.
Chapter 5

Hypomagnesemia in Leptospirosis
This chapter resulted in the following publications:


5.1 INTRODUCTION

The main aim of the work reported in this thesis was to investigate host responses in leptospirosis. In facilitating this aim, haematological and clinical biochemistry markers were investigated in leptospirosis patients. During the course of this work, it became apparent serum electrolyte concentrations such as chloride and potassium were routinely defined in leptospirosis (see Chapter 4) however, serum magnesium concentration was either not evaluated or evaluated and not reported. This was an important omission given that magnesium levels may effect a number of organ systems and mental status, and that altered mental status in leptospirosis is a poor prognostic indicator.

A number of studies have reported the clinical and laboratory findings observed in leptospirosis (Craig et al., 2009a; Esen et al., 2004) however, most studies have failed to report on the serum magnesium status of their patients. Craig et al. (2009b) suggested that serum magnesium status is under utilised by the medical community with only 5.4% of leptospirosis patients having their serum magnesium status determined at initial presentation. Magnesium is the fourth most abundant cation in the body and plays a diverse role in the physiology of many organ systems in the body (Swaminathan 2003). Studies that have reported on the serum magnesium status of leptospirosis patients have mixed findings. Early reports from Brazil revealed that leptospirosis patients had higher serum magnesium levels than healthy controls (de Jorge, 1970). Conversely, a recent report from Thailand revealed hypomagnesemia in 50% of leptospirosis patients and a case study reported
on an acute leptospirosis patient who displayed severe hypomagnesemia and required large doses of magnesium replacement during the acute phase (Khositseth et al., 2008; Spichler et al., 2008). This study aimed to add to the current limited understanding of hypomagnesaemia in leptospirosis by determining if leptospirosis patients experience hypomagnesemia during the acute phase of their disease.
5.2 PATIENTS AND METHODS

The study protocol was approved by the Human Ethics Committee from Queensland Health Forensic and Scientific Service (Approval Number 08-001/12) and the Human Ethics Research Committee from the University of the Sunshine Coast (Approval Number A/08/155). A total of 15 leptospirosis patients between the ages of 20 and 76 years were identified and investigated retrospectively using the leptospirosis patient database at the WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis. Of the 15 cases investigated, all displayed significant respiratory distress as evidenced by dyspnea, haemoptysis, diffuse alveolar haemorrhage and or acute liver or renal failure. All 15 patients eventually recovered from their infection. Leptospirosis was confirmed when leptospires were isolated from blood cultures in Ellinghausen-McCullough-Johnson-Harris (EMJH) media or detected in serum by real time PCR. Leptospirosis was also confirmed serologically if there was a greater than, or equal to, 4 fold increase in microscopic agglutination titre on follow up from the initial presentation, or an MAT titre ≥ 400. Pathology results were interrogated from the first samples taken following initial presentation and followed for 10 days.
5.3 RESULTS

The review of the patient’s serum magnesium status is shown in Table 5.1. The data in Table 5.1 reveals that in 14 of the 15 patients, during the first 10 days of presenting with leptospirosis, developed hypomagnesemia and this finding is consistent regardless of patient age or the infecting serovar.
Table 5.1. Patient Data.

* This patient did not have serum magnesium determined at initial presentation. The next day following presentation serum magnesium was determined and the patient had a serum magnesium concentration of 0.81 mmol / L (Normal reference range = 0.70-1.10 mmol / L).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Serum $[\text{Mg}^{2+}]$ mmol / L at Presentation</th>
<th>Hypomagnesemia (&lt; 0.70 mmol / L) at Day</th>
<th>$[\text{Mg}^{2+}]$</th>
<th>Infecting Serovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>Not Requested</td>
<td>1</td>
<td>0.62</td>
<td><em>L. borgpetersenii</em> serovar Hardjo</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>0.67</td>
<td>At presentation</td>
<td>0.67</td>
<td><em>L. borgpetersenii</em> serovar Hardjo</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>Not Requested</td>
<td>1</td>
<td>0.67</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>0.69</td>
<td>At presentation</td>
<td>0.69</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>Not Requested</td>
<td>1</td>
<td>0.67</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>0.68</td>
<td>At presentation</td>
<td>0.68</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
<td>Not Requested</td>
<td>Following initial presentation</td>
<td>0.61</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>0.69</td>
<td>At Presentation</td>
<td>0.69</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>0.79</td>
<td>2</td>
<td>0.69</td>
<td><em>L. interrogans</em> serovar Australis</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>0.79</td>
<td>1</td>
<td>0.59</td>
<td><em>L. interrogans</em> serovar Copenhageni</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>Not Requested</td>
<td>6</td>
<td>0.56</td>
<td><em>L. interrogans</em> serovar Zanoni</td>
</tr>
<tr>
<td>12</td>
<td>76</td>
<td>0.74</td>
<td>8</td>
<td>0.66</td>
<td><em>L. interrogans</em> serovar Zanoni</td>
</tr>
<tr>
<td>13</td>
<td>24</td>
<td>Not Requested</td>
<td>10</td>
<td>0.65</td>
<td><em>L. interrogans</em> serovar Zanoni</td>
</tr>
<tr>
<td>14</td>
<td>20</td>
<td>0.48</td>
<td>At Presentation</td>
<td>0.48</td>
<td><em>L. interrogans</em> serovar Zanoni</td>
</tr>
<tr>
<td>15</td>
<td>24</td>
<td>Not Requested</td>
<td>Did not develop hypomagnesemia *</td>
<td>-</td>
<td><em>L. interrogans</em> serovar Zanoni</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

Hypomagnesemia in critically ill patients can be as high as 65% and hypomagnesemia in intensive care patients has been associated with increased mortality (Swaminathan, 2003). The aim of the current chapter was to add to the currently limited understanding of hypomagnesemia in leptospirosis by determining if leptospirosis patients experience hypomagnesemia during the acute phase of their disease. The results of the current study are consistent with a recent study from Thailand reporting hypomagnesemia in 50% of patients with leptospirosis (Khositseth et al., 2008). The results of the current study are also consistent with the case report of a 15 year old female with leptospirosis who presented with a serum magnesium concentration of 0.56 mmol/L which dropped to 0.038 mmol/L on day 3 post admission despite receiving 6 g/day of magnesium therapy (Spichler et al., 2008).

Clinically, hypomagnesemia may result in muscle weakness and cramps, convulsions, atrial tachycardias, supraventricular and ventricular arrhythmias, electrolyte disturbances and altered mental status (Swaminathan, 2003). While the causes of hypomagnesemia include redistribution (from extracellular fluid into cells or bone), reduced intake, reduced intestinal absorption, and increased gastrointestinal loss, in leptospirosis it is most likely due to hypermagnesuria associated with the renal injury in general, and dysfunction or damage to the thick
ascending limb of the loop of Henle specifically since it is where 65-75% of filtered magnesium is reabsorbed (Swaminathan, 2003; Khositseth et al., 2008).

Although diabetes and genetic causes of hypomagnesemia (such as Bartter’s syndrome or Gitelman’s syndrome) or iatrogenic causes a such as furosemide use could not be determined from the records reviewed, this research adds to the slow accumulation of literature highlighting the importance of clinical monitoring of serum magnesium status in patients with leptospirosis especially since magnesium levels may affect mental status and that altered mental status in leptospirosis is a poor prognostic indicator.
Chapter 6

Consecutive episodes of Leptospirosis
This chapter resulted in the following publication:

6.1 INTRODUCTION

Leptospirosis is an important zoonotic disease caused by members of the genus *Leptospira*. Infections with pathogenic leptospires result in a spectrum of disease ranging from mild “flu like” illness to Weils’ disease. A high mortality is associated with the latter due to pulmonary haemorrhage and renal failure (Levett 2001). Levett (2003) correctly argues that cross reactions between serogroups and paradoxical reactions, where the initial immune response is directed toward a heterologous serovar confound the diagnostic process. Another confounding variable is original antigenic sin. Original antigenic sin was first posited by Francis (1953) and supported by others in the years that followed (Francis, Davenport & Hennessy 1955; Fazekas de St Groth & Webster 1966). This theory postulates that in serological investigations, the serotype with the highest neutralising titre observed after a secondary infection corresponds to the serotype that caused the primary infection. In the course of work presented in this thesis, data was discovered, relating to a patient who apparently experienced two episodes of leptospirosis (from two different serovars) over a period of three months and displayed peculiar serological results. On this basis and that there appear to be no other reports in the literature of host responses to consecutive infections, further investigation seemed warranted and consistent with the overall theme of the work reported in this thesis. Interestingly, the patient may have had a paradoxical reaction or alternatively had an additional leptospiral infection prior to presenting with the two infections.
6.2 CASE STUDY

This study was approved by the Human Ethics Committee from Queensland Health Forensic and Scientific Services (08-001/12) and the Human Ethics Research Committee from the University of the Sunshine Coast (A/08/155). The patient was a 33 year old male dairy farmer (a high risk occupation for leptospirosis) who reported exposure to rats (*Rattus norvegicus*), mice (*Mus musculus*), pigs (*Sus scrofa*), dogs (*Canis canis familiaris*), cats (*Felis domesticus*), bandicoots (*Isoodon obesulus*) and possums (*Trichosurus vulpecular*). From a returned questionnaire the attending medical officer reported that the patient presented with fever, headache, arthralgia, myalgia and conjunctival suffusion. At initial presentation, haematological and biochemical markers (Table 6.1 and 6.2) revealed that the patient was thrombocytopenic, lymphopenic, hyperkalemic and had elevated levels of aspartate transaminase (AST) and alanine transaminase (ALT). Blood cultures and serology excluded Flaviviruses and other organisms that commonly cause pyrexia in Australia. Analysis of the patient’s serum, using an enzyme linked immunosorbance assay (ELISA), did not detect anti-leptospiral IgM class antibody. Approximately four weeks after initial presentation, *Leptospira weilii* serovar Celledoni was isolated from one of the patient’s original blood cultures.

Ten days after initial presentation (two and a half weeks before the isolation of the serovar Celledoni from his blood culture) the patient continued to feel unwell and presented for a second time. In this instance, testing revealed that the patient was leptospiral IgM reactive by ELISA and had a dominant microscopic agglutination
test (MAT) titre of 1600 to *Leptospira kirschneri* serovar Bulgarica and a number of lower titre cross reactions (Table 6.3). At this stage, the patient’s MAT titre to serovar Celledoni equalled 100. Blood cultures were performed from samples taken at this second presentation however, as the patient was in the immune phase, no organisms were recovered.

Approximately three months after his initial presentation, the patient was ill and presented for a third time. At this third presentation, haematological and biochemical markers (Table 6.1 and 6.2) revealed that the patient was thrombocytopenic and lymphopenic. Again, blood cultures and serology excluded other organisms that commonly cause pyrexia in Australia. Testing this time revealed that the patient was leptosorial IgM reactive by ELISA and had a dominant MAT titre of 200 to serovar Celledoni and serovar Bulgarica. Three weeks after this third presentation *Leptospira interrogans* serovar Zanoni was isolated from the patient’s blood culture.
Table 6.1. Haematological markers from first presentation (acute phase) with *Leptospira weillii serovar Celledoni* and *Leptospira interrogans* serovar Zanoni infections.

<table>
<thead>
<tr>
<th>Haematological Markers</th>
<th>Normal Reference Range</th>
<th>Leptospira weillii serovar Celledoni infection</th>
<th>Leptospira interrogans serovar Zanoni infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/L)</td>
<td>135-185</td>
<td>168</td>
<td>151</td>
</tr>
<tr>
<td>White cell count (x 10⁹/L)</td>
<td>4-11</td>
<td>4.1</td>
<td>6.6</td>
</tr>
<tr>
<td>Neutrophils (x 10⁹/L)</td>
<td>2-8</td>
<td>2.94</td>
<td>5.48</td>
</tr>
<tr>
<td>Lymphocytes (x 10⁹/L)</td>
<td>1.2-4</td>
<td>0.73</td>
<td>0.79</td>
</tr>
<tr>
<td>Platelets (x 10⁹/L)</td>
<td>140-400</td>
<td>133</td>
<td>114</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.39-0.52</td>
<td>0.48</td>
<td>0.44</td>
</tr>
<tr>
<td>Red cell count (x 10¹²/L)</td>
<td>4.5-6</td>
<td>5.6</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Table 6.2. Clinical Chemistry markers from first presentation (acute phase) with *Leptospira weillii* serovar *Celledoni* and *Leptospira interrogans* serovar Zanoni infections.

<table>
<thead>
<tr>
<th>Biochemistry Markers</th>
<th>Normal Reference Range</th>
<th><em>Leptospira weillii</em> serovar <em>Celledoni</em> infection</th>
<th><em>Leptospira interrogans</em> serovar Zanoni infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mmol/L)</td>
<td>135-145</td>
<td>136</td>
<td>137</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>3.5-4.5</td>
<td>8.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>100-110</td>
<td>106</td>
<td>106</td>
</tr>
<tr>
<td>Anion Gap (mmol/L)</td>
<td>4-13</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>2.1-7.1</td>
<td>6</td>
<td>4.9</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>70-120</td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>60-83</td>
<td>73</td>
<td>60</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35-50</td>
<td>51</td>
<td>35</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>25-45</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Bilirubin (Total - µmol/L)</td>
<td>&lt; 20</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>53-128</td>
<td>63</td>
<td>42</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase (U/L)</td>
<td>&lt; 55</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>&lt; 45</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>&lt; 35</td>
<td>58</td>
<td>22</td>
</tr>
</tbody>
</table>
### Table 6.3. MAT / Serology results.*

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Species</th>
<th>Serovar</th>
<th>Strain used in MAT</th>
<th>First infection MAT Titre (Leptospira weilii serovar Celledoni isolated)</th>
<th>Second Infection MAT Titre (Leptospira interrogans serovar Zanoni isolated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australis</td>
<td>L. interrogans</td>
<td>Australis</td>
<td>Ballico</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Ballum</td>
<td>L. borgpetersenii</td>
<td>Ballum</td>
<td>Mus 127</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Bataviae</td>
<td>L. interrogans</td>
<td>Batavia</td>
<td>Swart</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>L. kirschneri</td>
<td>Bulgariaca</td>
<td>Nicolaev</td>
<td>1600</td>
<td>200</td>
</tr>
<tr>
<td>Canicola</td>
<td>L. interrogans</td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Celledoni</td>
<td>L. weilii</td>
<td>Celledoni</td>
<td>Celledoni</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>L. interrogans</td>
<td>Copenhageni</td>
<td>M20</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Cynopteri</td>
<td>L. kirschneri</td>
<td>Cynopteri</td>
<td>3522C</td>
<td>200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Djasiman</td>
<td>L. interrogans</td>
<td>Djasiman</td>
<td>Djasiman</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>L. interrogans</td>
<td>Grippotyphosa</td>
<td>Moskva V</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Sejroe</td>
<td>L. interrogans</td>
<td>Hardjo</td>
<td>Hardjoprajetno</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>Javanica</td>
<td>L. borgpetersenii</td>
<td>Javanica</td>
<td>Veldrat Batavia 46</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>L. interrogans</td>
<td>Kremastos</td>
<td>Kremastos</td>
<td>200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Sejroe</td>
<td>L. interrogans</td>
<td>Medanensis</td>
<td>Hond HC</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Panama</td>
<td>L. noguchii</td>
<td>Panama</td>
<td>CZ 214</td>
<td>&lt;50</td>
<td>50</td>
</tr>
<tr>
<td>Pomona</td>
<td>L. interrogans</td>
<td>Pomona</td>
<td>Pomona</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>L. interrogans</td>
<td>Robinsoni</td>
<td>Robinsoni</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Shermani</td>
<td>L. santarosai</td>
<td>Shermani</td>
<td>1342K</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Mini</td>
<td>L. interrogans</td>
<td>Szwajizak</td>
<td>Szwajizak</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>L. borgpetersenii</td>
<td>Tarassovi</td>
<td>Perepelitsin</td>
<td>100</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>L. interrogans</td>
<td>Zanoni</td>
<td>Zanoni</td>
<td>100</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

*The end point of the MAT reaction is deemed as the dilution of serum, which facilitates 50% agglutination, leaving 50% of leptospires free when observed under dark-field microscopy (Stallman, 1984). A titre ≥ 400 with a reactive IgM is indicative of a recent infection.
6.3 DISCUSSION

This is the first report of laboratory findings from a patient who suffered two different leptospiral infections in three months who may have had a third infection prior to both. The results showed that lymphopenia and thrombocytopenia are common in primary and secondary infections, while deranged liver function tests (ALT and AST) were observed only in the primary infection. The MAT titre of 1600 to serovar Bulgarica would, on *prima facie* evidence, suggest that this was the infecting serovar at the time of first presentation. However, the isolation of serovar Celledoni from the first presentation confounds this diagnosis. The doctrine of original antigenic sin postulates that the serotype with the highest neutralising titre after a secondary infection corresponds to the serotype that caused the primary infection. Based on this doctrine, the serological observations in this study suggest that the patient had an infection with serovar Bulgarica prior to becoming infected with serovar Celledoni and then serovar Zanoni. Interestingly, the MAT titres on the third presentation (when the patient was infected with serovar Zanoni) revealed that the serovar Bulgarica titre had dropped from 1600 to 200 and the serovar Celledoni titre had increased from 100 to 200. Consistent with original antigenic sin, the serovar Celledoni titre may have been attenuated because lymphocyte responses directed toward the prior serovar Bulgarica infection may have diluted the immune response to the serovar Celledoni infection. This is congruent with Mongkolsapaya *et al.* (2003) who submit that in Dengue infections, original antigenic sin in lymphocytes may suppress or delay viral elimination engendering higher viral loads and increased pathology. Alternatively, the initial reaction to serovar Bulgarica may have been a
paradoxical reaction which implies that there was no serovar Bulgarica infection prior to the two episodes of leptospirosis. Since serovar Zanoni was isolated from a blood culture taken during the third presentation, when the patient was still in the acute/leptospiremic phase of the disease, the patient had not yet developed antibodies to serovar Zanoni. Unfortunately, the patient was lost to follow up and the laboratory was unable to obtain a sample during the immune phase of the serovar Zanoni (final) infection. Regardless, the data above highlights the arduous task faced by reference laboratories in performing leptospiiral serological investigations.
Chapter 7

Leptospirosis and Goodpasture’s Syndrome
This chapter resulted in the following publications:


7.1 INTRODUCTION

Stanton and Tange (1958) described nine patients with a pulmonary-renal disorder that they called Goodpasture’s syndrome, after the author of an earlier report on the illness (Goodpasture, 1919). Goodpasture’s syndrome, now known to be a severe auto-immune disease causing glomerulonephritis, with or without pulmonary haemorrhage, is rapidly fatal without aggressive medical intervention. Underpinning the development of Goodpasture’s syndrome is the production of immunoglobulins targeted against type-IV collagen. These immunoglobulins have a high affinity for the glomerular basement membrane (GBM) of the kidney and the alveolar basement membrane of the lung (Hudson et al., 2003). Unfortunately, the exact aetiology or aetiologies of Goodpasture’s syndrome are unknown. In leptospiral infections of guinea pigs, it has been demonstrated that there is a deposition of IgM, IgG, IgA and complement C3 along the alveolar basement membrane, in a fashion similar to that seen in Goodpasture’s syndrome (Nally et al., 2004). It has also been reported that the renal lesions seen in leptospirosis can be immunologically mediated (Lai et al., 1982).

The possible production of auto-immune antibodies by the host during leptospirosis is an important avenue of any investigation of host responses in leptospirosis since associations between auto-immune antibodies or auto-immune disease and leptospirosis have been reported (Constantin et al., 1996; Daher et al., 2002; Tattevin et al., 2003; Barnabe and Fahlman 2008). However, at the time of writing, no study has investigated the possibility of the host producing auto
antibodies against Type 4 collagen, otherwise known as the Goodpasture’s syndrome antigen. This is a glaring knowledge gap given that, as shown in Chapter 4 of this thesis, lymphocyte numbers are more likely to return to normal during the immune phase of the disease and as reported in Chapter 1, lymphocytes are pivotal in the production humoral (antibody) responses. These antibody responses may include antibodies directed toward Type 4 collagen.

With this in mind, and given that marked renal and pulmonary dysfunction is common to both leptospirosis and Goodpasture’s syndrome, it was recently suggested that leptospires may be the aetiological agents of Goodpasture’s syndrome (Craig et al., 2009). This Chapter reports on work to investigate this hypothesis, by using an ELISA to check Leptospira-reactive sera from leptospirosis patients for antibodies that react with proteins found in human GBM.
7.2 PATIENTS AND METHODS

Overall, 40 leptospirosis patients were identified using the leptospirosis-patient database at the WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis at Cooper’s Plains (Queensland, Australia) and investigated retrospectively. Leptospirosis was confirmed when leptospires were either isolated from blood cultures in Ellinghausen-McCullough-Johnson-Harris medium or detected, by real-time PCR, in serum. The disease was also confirmed serologically if there was a rise of at least 4-fold in microscopic-agglutination-test (MAT; Faine, 1982) titre between presentation and convalescent follow-up or an MAT titre of > 400. Other common diseases causing pyrexia in Australia were excluded by serological testing and/or blood culture. Infections with dengue virus, Ross River virus, Barmah Forest virus, *Rickettsia* spp. and *Orientia* spp., for example, were excluded by serology. Negative blood cultures excluded infections with pathogenic *Staphylococcus* spp., *Streptococcus* spp., *Meningococcus* spp., *Pseudomonas* spp., *Haemophilus* spp., and anaerobic bacteria. To ensure that the sera screened in the anti-GBM ELISA came from patients in the immune phase of leptospirosis, only sera that were *Leptospira*-reactive, as evidenced by MAT titres of > 800, were investigated. Each such serum was diluted 1:50 before being tested in a commercial anti-GBM ELISA (Immunoscan Anti-GBM; Euro-Diagnostica, Malmö, Sweden) according to the manufacturer’s instructions. The calibration controls supplied by the manufacturer were used to produce standard curves of titre (measured in ‘ELISA units’) versus absorbance at 405 nm. Test samples giving titres of <10, 10–20 and >20 ELISA units were considered ‘negative’, ‘equivocal’ and
‘positive’, respectively. The study protocol was approved by the Human Ethics Committee of the Queensland Health Forensic and Scientific Service (approval 08-004/14).
7.3 RESULTS

The results from the anti-GBM ELISA are presented, with the corresponding results from the leptospirosis MAT in Table 7.1. These data indicated that none of the 40 patients in the immune phase of leptospirosis who were investigated had significant titres of antibodies against the antigen used in the anti-GBM ELISA, regardless of the infecting serovar involved.
Table 7.1. MAT and corresponding Anti-GBM ELISA results for the sera of 40 leptospirosis patients in the immune phase of the disease.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Leptospiral MAT Titre</th>
<th>Serovar</th>
<th>Anti-GBM ELISA IU/mL</th>
<th>Anti-GBM ELISA interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1600</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
<td>Szwajizak</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>4</td>
<td>1600</td>
<td>Australis</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>5</td>
<td>1600</td>
<td>Australis</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>6</td>
<td>1600</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>7</td>
<td>1600</td>
<td>Zanoni</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>8</td>
<td>3200</td>
<td>Australis</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>9</td>
<td>1600</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>10</td>
<td>800</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>11</td>
<td>&gt; 6400</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>12</td>
<td>800</td>
<td>Hardjo</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>13</td>
<td>1600</td>
<td>Szwajizak</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>14</td>
<td>3200</td>
<td>Arborea</td>
<td>&lt; 10</td>
<td>Non reactive</td>
</tr>
<tr>
<td>15</td>
<td>800</td>
<td>Australis</td>
<td>&lt; 10</td>
<td>Non reactive</td>
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GBM Positive Control: - - 42.2 REACTIVE
GBM Negative Control: - - <10 Non reactive
7.4 DISCUSSION

Associations between auto-immune antibodies or auto-immune disease and leptospirosis have been reported several times. Anti-neutrophil cytoplasmic antibodies (Constantin et al., 1996), anti-cardiolipin antibodies (Daher et al., 2002), anti-phospholipid syndrome (Tattevin et al., 2003) and reactive arthritis (Pappas et al., 2003), for example, have all been associated with leptospirosis. Of the leptospirosis cases investigated by Santiago et al. (2001), 23%, 10% and 17% carried anti-cardiolipin IgG, anti-cardiolipin IgM and anti-2-glycoprotein-I auto-antibodies, respectively. Antibodies directed toward the leptospiral LruA and LruB lipoproteins have been implicated in the auto-immune aspects of leptospiral uveitis (Verma et al., 2005), and sera from most (>65%) of the Indian cases of leptospiral uveitis recently investigated by Verma et al. (2008) were found to contain antibodies to these lipoproteins. Interestingly, Barnabe and Fahlman (2008) have delineated several clinical features that are shared by lupus and leptospirosis.

Given that combined renal and pulmonary dysfunction is common in both leptospirosis and Goodpasture’s syndrome, the aim of the present study was to test whether leptospires could be an aetiological agent of Goodpasture’s syndrome, as recently hypothesised by Craig et al. (2009). The present results (see Table) clearly fail to support this hypothesis where none of the 40 patients investigated had significant titres of antibodies that were reactive with the epitope, of human GBM, on which the ELISA used was based. The antigen for the ELISA is the M2 subunit from the non-collagenous (NC1) domain of the a-3 chain of type-IV collagen (Anon.,
2009). All patients with Goodpasture’s syndrome appear to have antibodies directed against this subunit (Wieslander and Heinegard, 1985), and the ELISA used in the present study is now routinely employed, in most auto-immune laboratories, as a serological test for Goodpasture’s syndrome.

Further work is now required to determine if leptospirosis is a risk factor in the development of any other pulmonary-renal syndrome associated with autoimmunity, such as Wegener’s granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, Behçet’s disease, IgA nephropathy or systemic lupus erythematosus.
Chapter 8

Conclusions
The advances this thesis has delivered to the medical community are significant and multifaceted. In examining host responses to leptospiral infections the first aim of this thesis was to investigate and determine if lymphopenia, if it did occur in leptospirosis, was serovar dependant. The data presented in Chapter 1 from a large sample size helped to reconcile the conflicting reports in the literature of whether lymphopenia is a common feature in leptospirosis. The results presented in Chapter 1 suggest that lymphopenia is a common feature of leptospirosis regardless of infecting serovar, the possible exception is serovar Cophenganei. In addition to resolving the previous lymphopenia paradox in the literature the data generated from the second aim revealed that lymphopenia was indeed common in the acute phase but less so in the immune phase. On the basis of the data presented in this thesis it was concluded that lymphopenia is a common phenomena in leptospiral infections however the phase of the disease (acute or immune) at which the patient first presents may affect the patients observed lymphocyte count.

The third aim of this thesis was determine if differences in laboratory markers exist to differentiate those with an uncomplicated episode of leptospirosis from those who were admitted to an intensive care unit with severe disease in an effort to identify leptospirosis patients who need more aggressive treatment regimens directed toward them. The data presented in chapter 3 revealed, when leptospirosis is suspected, patients presenting with any of the following; serum urea greater than 7.1 mmol/L, creatinine greater than 120 mmol/L, albumin less than 35 g/L, haemoglobin less than 135 g/L, haematocrit below 0.36, red cell count less than 4.5 x 10^9/L, platelet count less than 140 x 10^9/L, a white cell count greater than 11 x
$10^9/L$ and neutrophil count greater than $8 \times 10^9/L$ may need to be closely monitored and have aggressive treatment regimes directed toward them. These findings are significant and may provide front line emergency physicians with diagnostic keys that may ultimately save lives.

The fourth aim of this thesis was to monitor the magnesium status in leptospirosis patients during the acute phase (first 10 days of infection) to determine if they develop hypomagnesemia given the paradox in the research literature were some studies had observed it and others had not. The results from this aim have shown that more attention needs to be paid to the serum magnesium status in leptospirosis patients as 14 of the 15 patients followed displayed hypomagnesemia during the acute phase of the disease. This is significant because magnesium levels may affect mental status and that altered mental status in leptospirosis is a poor prognostic indicator in leptospirosis.

The fifth aim of this thesis was to determine if laboratory markers are different in consecutive infections, of the same host, with a different leptospiral serovars. Such a study is difficult and has eluded the research literature because of the difficulty with patient follow up and availability of diagnostic resources and expertise. The case study presented in this thesis, of a patient who had 2 confirmed (by culture isolation) leptospiral infections revealed that in a secondary infection lymphopenia and thrombocytopenia were evidenced and liver function tests (ALT and AST) were within the normal reference range. This study also revealed that
serological identification may be difficult because of paradoxical reactions or original antigenic sin.

The final aim of this thesis was to determine if leptospirosis patients produce auto-immune antibodies to type 4 collagen (the Goodpasture antigen) as associations between auto-immune antibodies or auto-immune disease and leptospirosis have been reported. However, prior to this thesis no study had investigated the possibility of leptospires as etiological agents of Goodpasture’s syndrome. This was perplexing given the closeness of the clinical presentation of both diseases. The results of the investigation of leptospires as etiological agents of Goodpasture’s syndrome revealed that the host does not produce auto-immune antibodies directed toward type 4 collagen.
Chapter 9

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Appendix A

Ethics Approvals
Appendix B

Publications
Appendix C

Statement of Intellectual Contribution for Manuscripts

Scott Craig and supervisors Associate Professor Glenn Graham and Dr David McKay conceptualised the experiment. Scott Craig gathered and analysed data, carried out the literature review and drafted, edited and revised the manuscript. All the authors were involved in editing, and revising the article for its scientific and medical content. All authors read and approved the final manuscript.


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